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Expression of miR-31 in rectal cancer patients and its effect on proliferation ability of rectal cancer cells SW837

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Abstract. – OBJECTIVE: This study aimed to investigate the expression of miR-31 in rectal cancer patients and its effect on the proliferation and invasion ability of human rectal cancer cells SW837.

PATIENTS AND METHODS: 55 rectal cancer cancerous tissue specimens and 55 corresponding adjacent tissue (tissue adjacent to carcinoma) specimens were collected from rectal cancer patients treated in The First Hospital of Jilin University from March 2014 to March 2015. Real Time-quantitative Polymerase Chain Reaction was used for detecting the expression level of miR-31 in cancerous tissue and corresponding adjacent tissues. Differences in the expression of miR-31 were compared between the two groups. Different miR-31 expression vectors were established and rectal cancer cells SW837 were transfected. MTT was used for detecting the proliferation ability of the cells in the miR-31-mimics group, miR-31-inhibitor group and miR-control group.

RESULTS: The expression level of miR-31 was significantly higher in rectal cancer tissues than that in the adjacent tissues ($p<0.05$). The expression of miR-31 was higher in the miR-31-mimics group (23.6±4.6) than that in the miR-control group (1.63±0.65), while the expression of miR-31 was lower in the miR-31-inhibitor group (0.65±0.23) than that in the miR-control group. The proliferation ability of cells at the 6th, 12th, 24th, 48th, and 72nd hours was higher in the miR-31-mimics group than in the miR-31-inhibitor group, while that of cells was significantly lower in the miR-31-inhibitor group than in the miR-control group, with statistically significant differences ($p<0.05$). The number of invasive membrane cells (cell membrane number) counted under a microscope was (84.2±10.6) cells in the miR-31-mimics group, (12.3±4.1) cells in the miR-31-inhibitor group, and (45.2±10.6) cells in the miR-control group. The invasion ability in vitro of SW837 cells significantly increased after the overexpression of miR-31 ($p<0.05$).

CONCLUSIONS: miR-31 is increasingly expressed in rectal cancer. Low expression of miR31 can inhibit the proliferation and invasion ability of the cells. MiR-31 is expected to become a current biotherapeutic target.

Key Words:
MiR-31, Rectal cancer, SW837, Proliferation.

Introduction

Rectal cancer is one of the most malignant tumors, with its mortality second only to that of liver and lung cancers. Males have higher mortality of rectal cancer than females. Approximately more than 1 million people are diagnosed with rectal cancer every year around the world and approximately over 700,000 rectal cancer patients die[1]. The onset of rectal cancer is occult. When patients have hemafecia, changes in bowel habits or defecate deformation and other symptoms, which suggest that they go to the hospital for treatment, they have been in the middle and late stage or have metastasis. As a result, they lose the best time for treatment. Rectal cancer seriously threatens human health and quality of life[2]. Its high-risk factors include age, family history, obesity, alcoholism, smoking, inflammatory bowel diseases, eating too few fresh vegetables and cellulosic foods, eating too much red and processed meat, high-fat diets, sedentary lifestyles and so on[3]. Invasion and metastasis are the main causes of death in rectal cancer patients. Early detection and treatment can greatly improve the 5-year survival rate of rectal cancer patients. It has been found that patients with visceral metastasis have a 5-year survival rate lower than 10.6%[4].

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Rectal cancer is still mainly treated by radical surgery. In recent years, targeted therapy has been widely used, which can prolong the survival time of patients. However, it has clear, unavoidable toxic and side effects, leading to its limited use[5]. High metastasis and recurrence rate are the main reasons for the high mortality in rectal cancer patients. However, clinically, the majority of patients undergoing radical surgery already have micro-metastasis that cannot be detected. This is also one of the main reasons for the failure of surgical treatment[6]. Found in Hela cells, miR-31 is located on chromosome 9p21.3, with a single seed sequence and high conservation in fruit fly and spinal animals[7]. It is highly expressed in liver cancer, lung cancer and other malignant tumors[8,9].

In this work, the expression of miR-31 in rectal cancer and its effect on the proliferation ability of rectal cancer cells SW837 were detected to analyze its regulatory effect on the proliferation and invasion of rectal cancer cells by rectal cancer cells SW837 expression.

Patients and Methods

Sample Collection

55 rectal cancer cancerous tissue specimens surgically resected and 55 normal fresh tissue specimens 50mm adjacent to carcinoma were collected from rectal cancer patients first diagnosed in The First Hospital of Jilin University from March 2014 to March 2015. Patients included 36 males and 19 females from 30 to 76 years old. Tissues were resected, stored in liquid nitrogen and then transferred to be stored in a refrigerator at -80°C for RNA extraction. All cancerous tissue specimens were diagnosed by pathology, adjacent tissues with no cancer cell and inflammatory cell infiltration. Rectal cancer tissues were diagnosed and staged according to the 7th edition of the TNM staging criteria of AJCC (American Joint Committee on Cancer)[10]. All patients had no history of tumors, liver and kidney dysfunction, abnormal bleeding or coagulation dysfunction. Patients had complete medical records and follow-up data. Patients did not receive systematic drug treatment before the operation. This study was approved by the Ethics Committee of The First Hospital of Jilin University. All patients and their families were informed and signed an informed consent.

Reagents and Instruments

SW837 cells and human rectal mucosal epithelial cells were purchased from Wuhan Technology Co., Ltd. (Wuhan, China), TransScript Green miRNA Two-Step qRT-PCR SuperMix kit from TransGen Biotech (Beijing, China). Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA), fetal bovine serum (FBS), penicillin-streptomycin double antibody, Lipofectamine^{TM} 2000, TRIzol reagent, transwell cell invasion kit from Thermo Fisher Scientific (Waltham, MA, USA), MTT cell proliferation kit from Shanghai Biotechnology (Shanghai, China). Primers for miR-31 were designed and synthesized by Shanghai Jima Co., Ltd (Shanghai, China), see Table I; PCR instrument from Thermo Fisher Scientific, (Waltham, MA, USA) and Labserv K3 microplate reader from Woyuan Technology Co., Ltd., (Shanghai, China).

Cell Culture

SW837 cells were cultured (37°C, 5% CO_2 constant temperature incubator) in a RPMI-1640 medium (10% FBS, penicillin-streptomycin double antibody). Cells in the logarithmic growth phase were subjected to subsequent experiments after the culture. The concentration of cells in the logarithmic growth phase was adjusted to $1*10^6$ cells/well in a 6-well plate and transfected according to the Lipofectamine^{TM} 2000 kit instructions. The miR-31-mimics group, the miR-31-inhibitor group and the miR-control group were set up in the experiment.

MTT Detection of Proliferation In vitro of Rectal Cancer Cells

Rectal cancer cells were prepared into the single-aligned cell suspension, and incubated in a 96-well plate, with 2×10^3 cells in each well. MTT was used for detecting cell viability. During the detection, 10 μL of 5 mg/ml MTT solution was added to each well, continuously incubated in the incubator for 4h. 150 μL of dimethyl sulfoxide (DMSO) was added to each well, shaken at room

Table I. Primer sequences.

Gene	Upstream primer	Downstream primer
miR-31	5'-ACGCGGCAAGATGCTGGCA-3'	5'-CAGTGCTGGGTCCGAGTGA-3'
U6	5'-TGGAACGATACAGAGAAGATTAGCA-3'	5'-AACGCTTCACGAATTTGCGT-3'

temperature for 15 min. The crystals were dissolved completely under the light microscope. ELISA (Enzyme-linked immunosorbent assay) was used for detecting the OD value at a wavelength of 570 nm. The detection was repeated 3 times per well.

Cell Invasion Experiment

The cultured cells in each group were collected, incubated in a Matrigel-coated transwell chamber at 2×10^4 cells and cultured at 37°C for 24h. Cells were digested and resuspended in a serum-free medium RPMI-1640. After the cell concentration was adjusted to 1×10^5/mL, cells were incubated in the upper chamber, and the routine medium was added to the lower chamber as a chemokine. After culturing for 24h, the filter membrane was taken out, fixed with 4% paraformaldehyde and stained with Gimsa. Five layers in the membrane were randomly selected and the average value was obtained as the permeability value.

Real Time-Quantitative Polymerase Chain Reaction (qRT-PCR) Detection of Expression Level of MiR-31 in Cells

The collected cells and tissues were used for extracting total RNA with the TRIzol extraction reagent, the ultraviolet spectrophotometer and agarose gel electrophoresis for detecting the purity, concentration and integrity of the total RNA extracted, TransScript® miRNA RT Enzyme Mix and 2×TS miRNA Reaction Mix for the reverse transcription of the total RNA, with the procedure in strict accordance with the manufacturer's kit. The cDNA reversely transcribed was collected and stored, and another part was taken to continue subsequent experiments. The TransScript Green miRNA Two-Step qRT-PCR SuperMix was used for PCR amplification experiment. MiR-31 PCR reaction system was as follows: 1 μL of cDNA, 0.4 μL of each of upstream and downstream primers, 10 μL of 2 X TransStart® Top Green qPCR SuperMix, 0.4 μL of ROX Reference Dye II 50×, and ddH_2O finally added to 20 μL. MiR-31 PCR reaction conditions were as follows: pre-denaturation at 94°C for 30s, denaturation at 94°C for 5s, annealing at 60°C for 30s for a total of 40 cycles. Three replicate wells were set for each sample and the experiment was repeated 3 times. In this study, U6 was used as an internal reference, and $2^{-\Delta ct}$ was used for analyzing the data.

Statistical Analysis

SPSS 20.0 software package (SPSS IBM, Armonk, NY USA) was used for the statistical analysis, GraphPad Prism 7 (La Jolla, CA, USA) for plotting the data. Measurement data were expressed as mean ± standard deviation (Mean ± SD). Independent sample *t*-test was used for comparison between the two groups, which was denoted by t, analysis of variance for comparison among multiple groups, repeated measures analysis of variance for comparison at different time points, which was denoted by F, LSD-*t*-test for pairwise comparison in the group. When $p<0.05$, there was a statistically significant difference between the two groups.

Results

Expression of MiR-31 in Rectal Cancer Tissues and Adjacent Tissues

The expression of miR-31 was (4.81±0.36) in cancerous tissues of patients, markedly higher than (1.16±0.21) in adjacent tissues, with a significant difference (t=64.949, $p<0.001$) (Figure 1).

Relationship Between the Expression Level of MiR-31 and Clinicopathologic Features

The expression of miR-31 was not related to the gender, age, tumor volume and differentiation degree of patients, with no statistically signi-

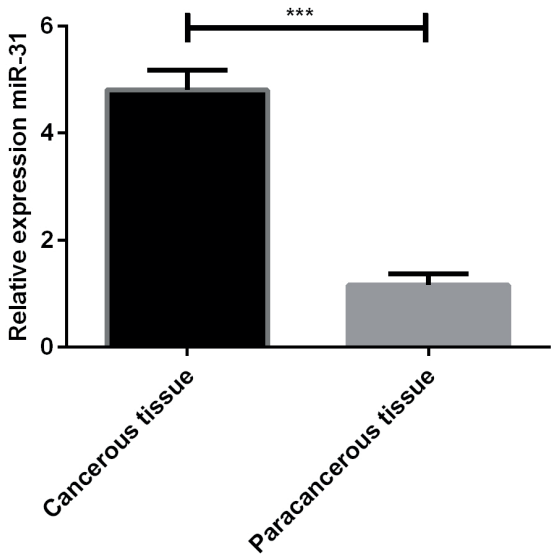

Figure 1. Expression of miR-31 in tissues of patients. qRT-PCR showed that the expression of miR-31 was significantly higher in the cancerous tissue of patients than that in the tissue adjacent to carcinoma, with a significant difference ($p<0.001$).

Table II. Relationship between expression level of miR-31 and clinicopathologic features.

Variables	No.	miR-319	t	p
Gender				
Male	36	4.26±0.24	1.151	0.137
Female	19	4.12±0.45		
Age				
≥55	30	4.28±0.82	0.959	0.342
<55	25	4.05±0.96		
Tumor volume				
≥5 cm	31	4.63±0.38	1.210	0.159
<5 cm	24	4.15±0.42		
Differentiation degree				
Poor and moderate	34	4.69±0.23	1.196	0.237
High	21	5.21±0.35		
Lymph node metastasis				
No	36	4.01±0.22	2.683	0.010
Yes	19	3.84±0.23		
Distant metastasis				
No	19	4.35±0.58	2.902	0.005
Yes	36	3.95±0.43		
Clinical staging				
I+II	23	3.69±0.26	2.256	0.028
III+VI	32	4.70±0.32		

ficant difference (p>0.05), but related to distant metastasis, lymph node metastasis and clinical staging, with a statistically significant difference (p<0.05). The expression of miR-31 was higher in stage III/IV of TNM staging than that in stage I/II, suggesting that the expression of miR-31 is a clinical indicator that predicts tumor malignancy and metastasis (Table II).

Expression of MiR-31 in Cells

PCR was used for detecting the expression of miR-31 in SW837 cells and human rectal mucosal epithelial cells. The expression of miR-31 was (1.702±0.095) in SW837 cells, significantly higher than (0.925±0.081) in human rectal mucosal epithelial cells, with a significant difference (t=10.780, p=0.001).

RT-PCR Detection of SW837 Cell Transfection

MiR-31-mimics, miR-31-inhibitor and miR-control were respectively transfected with SW837 cells for 48h and then lysed. The total RNA was extracted to detect the expression of mir-31 in cells and to determine the transfection effect. The transfection of cells in the three groups was compared. There was a significant difference in the expression of mir-31 among the miR-31-mimics group (3.824±0.284), the miR-control group (1.732±0.102) and the miR-31-inhibitor group (0.684±0.084) (F=234.432, p<0.001). The intra-group comparison showed that the expression of mir-31 was significantly higher in the miR-31-mimics group than that in the miR-control group and the miR-31-inhibitor group, with a difference (p<0.05). That of mir-31 was higher in the miR-control group than that in the miR-31-inhibitor group, with a difference (p<0.05) (Figure 2).

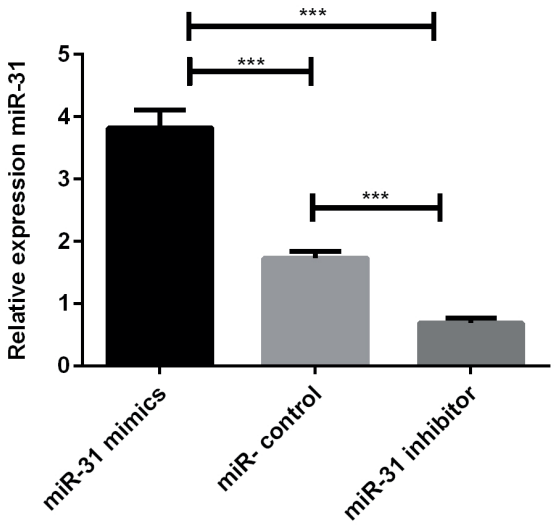

Figure 2. Expression in each group of cells after transfection. Cells in the three groups were transfected. PCR showed that the expression of miR-31 was significantly higher in the miR-31-mimics group than in the miR-control group and the miR-31-inhibitor group, with a difference (p<0.05). That of mir-31 was higher in the miR-control group than in the miR-31-inhibitor group, with a difference (p<0.05).

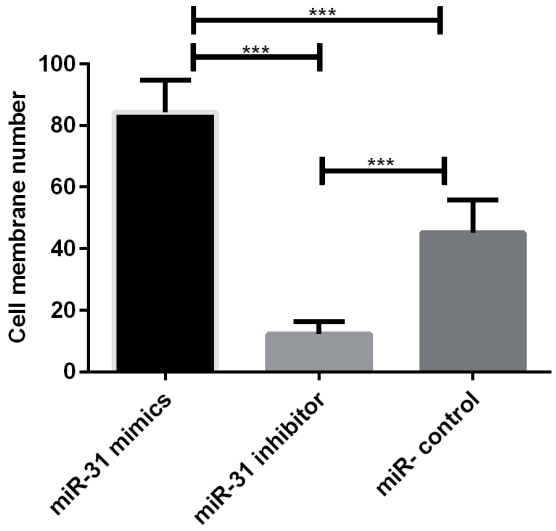

Figure 3. Invasion of cells in three groups. Transwell chamber was used for detecting the invasion ability of cells. The number of invasive membrane cells was significantly higher in the miR-31-mimics group than that in the miR-31-inhibitor and the miR-control groups, with a statistically significant difference (p<0.05), while that of invasive membrane cells was significantly higher in the miR-control group than that in the miR-31-inhibitor group, with a statistically significant difference (p<0.05).

MTT Detection of Effect of MiR-31 on the Proliferation Ability of SW837 Cells

MTT was used for detecting the growth of SW837 cells in different groups at the 6th, 12th, 24th, 48th, and 72nd hours. The results showed that the proliferation ability of cells in the miR-31-mimics, miR-control and the miR-31-inhibitor groups increased with time. However, the comparison among groups showed that there was no statistically significant difference at the 6th and 12th hours of proliferation (p>0.05). At the 24th hour, there was no difference between the miR-31-inhibitor group and the miR-control group (p>0.05), but there was a difference among other groups (p<0.05). There was a statistically significant difference between the two groups at each time point (p<0.05). The intra-group comparison showed that there was no difference between the 6th and 12th hours, between the 24th and 48th hours in the miR-31-mimics group (p>0.05), but there was a statistically significant difference at other time points (p<0.05). There was no statistically significant difference between the 6th and 12th, 24th hours, between the 12th and 24th hours, between the 48th and 72nd hours in the miR-control group (p>0.05), but there was a statistically significant difference at other time points (p<0.05). There was a difference between the 72nd and 6th, 12th hours in the miR-31-inhibitor group (p<0.05), but there was no statistically significant difference at other time points (p>0.05) (Table III).

Effect of MiR-31 on Invasion Ability of Rectal Cancer SW837 Cells

Transwell *in vitro* invasion experiment showed that cells in each group had penetrated the microporous membrane after 48h. The number of invasive membrane cells counted under the microscope was (84.2±10.6) cells in the miR-31-mimics group (12.3±4.1) cells in the miR-31-inhibitor group and (45.2±10.6) cells in the miR-control group, with a statistically significant difference (F=48.274, p<0.001). The comparison among groups showed that the number of invasive membrane cells was significantly higher in the miR-31-mimics group than in the miR-31-inhibitor group and the miR-control group, with a statistically significant difference (p<0.05), while that of invasive membrane cells was significantly higher in the miR-control group than in the miR-31-inhibitor group, with a statistically significant difference (p<0.05) (Figure 3).

Discussion

Rectal cancer is caused by environmental pollution, changes in dietary habits, inheritance and other factors, with its annual incidence on the

Table III. Detection of cell proliferation.

Group	6h	12h	24h	48h	72h
miR-31 mimics group	0.254±0.054	0.481±0.100	0.784±0.125$^{\circ\S}$	0.964±0.185$^{\circ\S}$	1.435±0.227$^{\circ\S+\wedge}$
miR-control group	0.235±0.082	0.284±0.102	0.430±0.115*	0.695±0.1625$^{*\circ\S}$	0.822±0.182$^{*\circ\S+}$
miR-31 inhibitor group	0.261±0.073	0.29±0.102	0.332±0.102*	0.421±0.152$^{*\#}$	0.538±0.177$^{*\#\circ\S}$

Note: *indicates a difference compared to the miR-31-mimics group (p<0.05). #indicates a difference compared to the miR-control group (p<0.05). §indicates a difference compared to the 6th (p<0.05). °indicates a difference compared to the 12th hour (p<0.05). +indicates a difference compared to the 24th (p<0.05). ^indicates a difference compared to the 48th (p<0.05).

rise[10,11]. It has posed a serious threat to human and public health. Colorectal adenoma is the precancerous lesion of rectal cancer. The expression level of genes abnormally changes during the progression of colorectal adenoma to rectal cancer[12,13].

MiRs have been a research hotspot in recent years. As a class of 19-22 nt non-coding short-stranded RNA, they are highly conserved and can bind to target gene 3'-UTRs through complete or incomplete complementary pairing, thereby being involved in the regulation of target genes[14]. Studies show that a variety of miRNA are closely related to the occurrence and development of tumors, which often play the role of tumor suppressor genes or cancer-promoting genes[15]. MiR-31, an important member of miRs, is differentially expressed in lung and gastric cancer, head and neck neoplasms and ovarian cancer[16-18]. Scholars[19,20] also show that inhibiting or promoting the expression of miR-31 can regulate the biological function of tumor cells.

In this work, the expression of miR-31 in rectal cancer tissues was first detected. The results showed that the expression of miR-31 was significantly higher in rectal cancer tissues than in adjacent tissues, indicating that miR-31 is differentially expressed in rectal cancer tissues. The expression of miR-31 in normal human rectal mucosal epithelial cells and rectal cancer SW837 cells was detected. The results showed that the expression of miR-31 was significantly lower in human rectal mucosal epithelial cells than in rectal cancer SW837 cells. Subsequently, the expression of miR-31 in patients' tissues and the clinical data were analyzed. The results showed that the expression of miR-31 was different in lymph node metastasis, distant metastasis and clinical staging. In the study by Bandrés et al[21], 10 rectal cancer specimens were detected. The results showed that patients with stage IV rectal cancer have a significantly higher expression of miR-31 in tissues than patients with stage II rectal cancer, which is a good testimony to our results. This also shows that involved in the occurrence and development of rectal cancer, miR-31 is expected to become a potential diagnostic indicator for clinical rectal cancer. However, the way in which miR-31 affects the growth and invasion of tumor cells is unclear. Therefore, different miR-31 expression vectors were transfected, and the over-expression group and the inhibition group were established. MTT and transwell chamber were used for detecting the proliferation and invasion ability of cells in each group. The results showed that the proliferation and migration ability of cells was higher in the miR-31-mimics group than those in other groups. The proliferation and migration of cells were inhibited by inhibiting the expression of miR-31 in cells, suggesting that the proliferation and migration of cancer cells can be effectively reduced by inhibiting the expression of miR-31 in cancer cells. Wang et al[22] observe that the proliferation and invasion of gastric cancer cells can be effectively inhibited by inhibiting the expression of miR-31 in gastric cancer cells. The experiments in this work have shown that miR-31 has the same function in rectal cancer cells. This indicates that miR-31 has the same effect in different tumors. Related studies[23,24] show that inhibiting the expression of miR-31 can reduce cell viability, which can induce apoptosis, block cell cycle and effectively inhibit cancer cell growth.

In this work, the expression of miR-31 in tissues of rectal cancer patients was detected. The results showed that miR-31 was highly expressed in colon cancer tissues, and it can effectively inhibit the proliferation and invasion of rectal cancer cells by inhibiting the expression of miR-31 in rectal cancer tissues. However, there are still limitations in this study. First, the downstream target genes are not predicted. The way in which miR-31 regulate the biological function of cells is still unclear. Secondly, the number of samples in this study is small, and no normal patient samples are collected. It is not clear whether it can be used as a diagnostic criterion for rectal cancer. Therefore, it is hoped to add more experiments in future researches and increase the sample size to support the results of this work.

Conclusions

We observed that miR-31 is increasingly expressed in rectal cancer. The decrease in its expression can inhibit the proliferation and invasion ability of cells. MiR-31 is expected to become a current bio-therapeutic target.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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